Acute liver injury induced by carbon tetrachloride and demonstration of hepatoprotective and *in-vitro* anti-oxidant capacity by *Piper nigrum* extracts and pure compound – piperine in Swiss Albino rats.

Farhana Tasleem[1*], Saad Bin Zafar Mahmood[2], Iqbal Azhar[3], Zafar Alam Mahmood[4]

[1, 3, 4] Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Karachi- 72570, Pakistan.

[2] Department of Medicine, The Aga Khan University Hospital, Stadium Road, Karachi-Pakistan

* Corresponding Author:
Farhana Tasleem
Department of Pharmacognosy, Faculty ofPharmacy and Pharmaceutical Sciences,
University of Karachi, Karachi- 72570, Pakistan

ABSTRACT

Background: The objective of present study was to evaluate the hepatoprotective effect against liver injury induced by CCl_4 as well as i*n-vitro* anti-oxidant property assessed by reducing power capacity (RPA) of hexane, ethanol extracts along with pure compound piperine, extracted from fruits of *Piper nigrum* L.

Methodology: This pre-clinical animal study estimates the prophylactic and therapeutic hepatoprotective effect of hexane and ethanol leaves extracts along with pure compound piperine extracted from fruits of *Piper nigrum*. Both sexes of albino rats were distributed into a normal control group, a CCl_4 hepatotoxicity control group (receiving single oral dose of CCl_4 1.5 ml/kg body weight) in equal volume of olive oil (1:1) and nine treatment groups receive hexane and ethanol leaves extracts (75, 150, 250 mg/kg body weight) and pure compound piperine (25, 75, and 100 mg/kg body weight) along with toxicant CCl_4. Standard drug (Betaine Glucuronate + Diethanolamine

Glucuronate + Nicotinamide Ascorbate) treated group (5.37, 10.75, 16.13 mg/kg body weight) along with toxicant CCl_4. Orally for five consecutive days. Liver damage was assessed by the biochemical estimation of serum activities of hepatic enzymes Alkaline phosphatese (ALP), Alanine aminotransferase (ALT), Gamma-*glutamyl transferase* (*γ-GT*), Direct bilirubin (DB) and Total Bilirubin (TBL). These tests are referred to as liver function test (LFT). This investigation test was supported by histopathological examinations. While tested extracts and compound were also analyzed for anti-oxidant capacity using reducing power ability (RPA). The reducing power assay applied to regulate the potential of an anti-oxidant to donate an electron.

Results: The estimation of hepatoprotective effect of test samples showed a potential of liver protection both in prophylactic and therapeutic model in a dose dependent manner against liver injury induced by CCl_4. But more significant results of enzymes suppression were seen in prophylactic model by piperine at a dose of 100 mg/kg body weight. This was an effective investigation test with high negative prognostic value of liver diseases that were supported with histopathological examination of rat liver section. Moreover, Piperine exhibited the highestreducing power capacity measured by RPA assay among all tested samples but less than standarddrug ascorbic acid.

Conclusion: *Piper nigrum* L. fruit can be used as an effective hepatoprotective agent both in prophylactic and therapeutic models along with antioxidant potential.

Keywords: Anti-oxidant, Biochemical parameters, Carbon tetrachloride, Hepatoprotective, Histopathological examination.

INTRODUCTION:

Liver have strong potential of detoxification against toxic agents and synthesize the health beneficent substances. It also controls the biochemical pathways associated to growth, immune

system, nutrient supply and reproduction in the body. Human beings are generally subjected to different type of chemical substances and these toxic substances generate free radical which modify lipid per oxidation and disrupt the bio membrane and damage cells and tissues [1]. CCl_4 is regularly used as a potent hepatotoxin. Oral administration of CCl_4 causes acute liver damage and effect the liver function that may leads to damaging of hepatocellular membrane. Degree of hepatic damage is analyzed by elevated levels of marker serum enzymes mainly ALP, ALT, γ- GT, DB and TBL. CCl_4 induced toxicity cause lipid per oxidation by activating the metabolic enzyme cytochrome P450 in the endoplasmic reticulum to generate trichloromethyl free radical (CCl_3). Consequently it combined with cellular lipids and protein in the presences of oxygen. These changes in structure of endoplasmic reticulum may lead to liver damage [2, 3].

Piper nigrum L. has many therapeutic properties and mainly cultivated for its fruits used as spice. *Piper nigrum,* as a phytopharmaceuticals, included in Ayurvedic and traditional natural prescriptions and recommended in the management and supportive treatment of cough, cholera, dyspepsia, gastric ailments and arthritic disorders. Pharmacological investigations of fruit indicated anti-inflammatory, anti-oxidant, anti-helminthic, and anti-asthmatic properties, justifying its use in the management of pain, piles, and cough as indicated in traditional system of medicine [4, 5]. Documented phytochemical investigation revealed the presences of diverse bioactive secondary metabolites such as alkaloids, glycosides, steroids, tannins, phenol, flavonoids, steroids and resins. However therapeutic activity depends on the classes and mostly the concentration of bioactive compounds. Pungent active alkaloid piperine the major chemical pure compound extracted from ethanol extract of *Piper nigrum* fruits play a significant role in diet and medicine. Phenolic compounds possess one or more aromatic ring with one or more hydroxyl group and associated with various physiological properties such as anti-inflammatory, anti-microbial and anti-oxidant effect [6].

Based on the *Piper nigrum* early studies background, the aim of the research was designed to

investigate the possible hepatoprotective potential of hexane; ethanol extracts along with pure isolated compound piperine from fruits of *Piper nigrum* through anti-oxidant mechanism of action of phytoconstituents in pathogenesis of CCl_4 induced hepatotoxicity.

METHODS

Chemicals, drugs and diagnostic kits

All chemicals and solvents used in the study were of analytical grade. Potassium ferric cyanide from Dae-Jung, trichloroacetic acid from AnalaR and Ferric chloride from Oxoid was purchased. Carbon tetrachloride and Ascorbic acid were obtained from Sigma. While the standard drug Betaine Glucuronate + Diethanolamine Glucuronate + Nicotinamide Ascorbateand Pentothal sodium 500mg were purchased from local pharmacy. Analytical kits used for the estimation of serum ALP, ALT, γ- GT, DB and TB levels were purchased from Ecoline.

Plant material and extraction

The fruit of *Piper nigrum* was purchased from local market of Karachi, Pakistan and identified by Dr. M. Mohtasheem ul Hassan, The identified sample specimen code (*Piper nigrum*L PN-03-09) is available in Herbarium of the Department of Pharmacognosy University of Karachi, Karachi, Pakistan. Dried fruits of *Piper nigrum* (3 kg) were soaked in hexane for 15 days. After 15 days hexane extract was filtered. The residue was soaked in ethanol for another 15days, finally after 15 days ethanol extract was filtered and both the filtrates were evaporated under reduced pressure by using rotary evaporator at 45 ºC and 50 rpm. The hexane and ethanol extracts were stored in airtight and amber colored glass vials separately for biological studieswith proper identification. Both the extracts were solubilized in 10% dimethylsulfoxide for oral administration to the test groups of animals at different doses according to the body weight.

Isolation of pure compound piperine

Method used, already documented in an earlier research publication [5, 7].

Phytochemical screening of secondary metabolites

Ethanol extract of *Piper nigrum* fruits was assessed for preliminary phytochemical screening. The standard protocols were followed to identify the phytoconstituents [8, 9, 10, and 11].

Anti-oxidant assay

For analysis of *in-vitro* anti-oxidant activity, 100 mg of pure compound piperine, ethanol extract, hexane extract and Ascorbic acid (standard drug) were taken in 50 ml volumetric flask and dissolved with sufficient amount of solvent (ethanol + water, 1:1). The volume was raised upwith as stock solution having 2000 µg/ml. Four different concentrations 1000 µg/ml, 500 µg/ml, 250µg/ml, 100 µg/ml pure compound, hexane extract, ethanol extracts and standard drug were prepared from stock solution. Each concentration of pure compound, extracts and standard drug (1 ml) was taken in test tube. To the test tube 5 ml of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferric cyanide solution was added and mixed well. This mixture was incubated at 50°C for 20 minutes. After incubation, to stop the reaction 5 ml tri-chloroacetic acid was added and then kept for centrifugation at 3000 rmp for 10 minutes. After centrifugation 5 ml of supernatant was collected into one more test tube. In the supernatant 5 ml of distilled water and 1 ml of ferric chloride (0.1%) were added. After 10 minutes absorbance was measured at 700 nm on spectrophotometer. The reducing power is linearly proportional to the concentration of thesample. Whole experiment was conducted in triplicate. Control sample was prepared by adding all reagents but without test sample [12, 13]. The percentage of reduction power of test samples compared to standard (Ascorbic acid) was calculated by following formula [14].

$$(\%) \text{ of reduction power} = [1- (1- A_S/ A_C)] \times 100$$

A_S = absorbance of sample

A_C = absorbance of standard at maximum concentration tested

Animals

Swiss Albino Rats of both sexes weighing 150 – 200g were used in this study. Animals were kept in individual stainless steel cages and maintained under laboratory condition of temperature (23±3ºC) with 12/12h light and dark cycles and were allowed free attain of food and water and marked with their identification. The experimental procedures were approved by Board of Advanced Studies and Research, University of Karachi BASR/No./0265/ Pharm.

Experimental design/ protocol

The rats were divided into six major groups i.e. A, B, C, D, E and F, each group included five animals (n=5). Pure compound piperine was administered orally to group 1-A, 2-A, 3-A at three different doses i.e. 25, 75, and 100 mg/kg body weight. While hexane and ethanol extracts were delivered orally to group 1-B, 2-B, 3-B, and 1-C, 2-C and 3-C at doses of 50,100 and 150 mg/kg body weight accordingly. Group D was served as a standard group and treated with standard drug 5.37, 10.75, 16.13 mg/kg body weight to group 1-D, 2-D and 3-D respectively. Group Ewas exposed with single oral dose of 1.5 ml/kg body weight CCl_4 in equal volume of olive oil (1:1) and assigned as toxicant group. Group F was given saline presented as control group. The study was performed in duplicate i.e. prophylactic and therapeutic models keeping all the parameters same as described above.

Induction of hepatotoxicity

Scanning of hepatoprotective activity of extracts and pure compound were performed in two models [2].

Prophylactic study model

This study model was conducted by pretreatment of test samples in rats, to inhibit the liver injury by toxicant. The rats were divided into pre-defined groups (experimental design). They were given their specific doses for five days constantly, after the half an hour of administration of last dose of test samples on day five they were toxicant by oral administration of CCl_4 except the control group.

Therapeutic study model

In this model of analysis, rats were firstly received oral single dose of CCl_4. After half an hour of CCl_4 administration animals were subjected to their pre-defined doses (experimental design) of test samples for 5 days regularly excluding control group. On day six rats of both study models were sacrificed using anesthesia and approximately 5 ml blood samples from each animal were drawn by cardiac puncture using heparinized microcapillary tubes. Incised the abdomen of animals and liver was eradicated.

Serum preparation

Blood samples were allowed to coagulate at room temperature for half an hour and were centrifuged at 2500 rpm for 15 minutes. Clear serum was separated for analysis of biochemical parameters (ALT, ALP, γ-GT, DB and TB).

Liver tissue preparation

Hepatic lobes were used for the preparation of histopathological slides. Liver tissues were

fixed with 10 % phosphate-buffered neutral formalin, dehydrated in graded (50–100 %) alcohol and embedded in paraffin. Thin sections were cut and stained with hematoxylin and eosin stain for microscopic assessment. The initial examination was qualitative, with the purpose of determining histopathological lesions in liver tissue [15].

Biochemical analysis

Liver damage was assessed by the estimation of serum activities of hepatic ALP, ALT, γ- GT, DB and TB according to the reported methods by Edwards and Bouchier [16] using commercially available kits. The enzyme activities were measured and read on a Photometric Micro lab. (17). Liver function test was performed at 25℃, and all the reagents are ready to use.

Hepatic scoring to evaluate liver pathology

Laboratory evaluation of liver damages reviewed and scored as hepatic fibrosis, congestion, hepatocellular necrosis and vascular features via biopsy specimen of liver tissues [18, 19]. The detection of degree of fibrosis was based on the observation of various staging (Table 1), which ultimately determines a distinctive phase or stages necrosis or in a broader sense the damages occur in liver cells. Similarly the grading of inflammation condition of the portal tract was recorded on the basis of lobular damages observed in the portal vein (Table 2)

Table 1. Criteria used to detect staging of fibrosis in portal tract

Staging	Descriptive	Criteria
0	No fibrosis	Normal connective tissues
1	Portal fibrosis	Fibrous portal expansion
2	Periportal fibrosis	Pericentral and septal fibrosis; no bridging fibrosis
3	Septal fibrosis	Fibrous septa with architectural distortion ; no obvious cirrhosis (bridging fibrosis)
4	Cirrhosis	Cirrhosis, complete nodule formation

Table 2. Criteria used to detect grading of inflammation in portal tract

Grading	Descriptive	Criteria
		Lobular inflammation and Necrosis
0	Portal inflammation only; no activity	None
1	Minimal	Minimal; occasional spotty necrosis
2	Mild	Mild; little hepatocellular damage
3	Moderate	Moderate; with noticeable hepatocellular change
4	Severe	Severe; with prominent diffuse hepatocellular damage

Statistical analysis

All values were given in mean ± SEM. The biochemical data were analyzed by student's t-test followed by SPSS software version 20. Statistical significance level was set as *P<0.001 and **P<0.01 significant as compared to toxicant.

RESULTS

Phytochemical screening

Ethanol extract of *Piper nigrum* fruits confirmed the presence of alkaloids, glycosides, steroids, tannins, phenol compounds, flavonoids, steroids and resins. These findings were similar

to the previous phytochemical screening results conducted by Ganesh [20].

Anti-oxidant Assay

Correlation of anti-oxidant activity with reducing power of pure compound piperine, hexane and ethanol extracts of *Piper nigrum* fruits were given in (Table 3 and 4). The reducing power of test samples increased with an increased in the concentrations and were recorded in the order of pure compound piperine (1.039±0.015 and 72.10%) > hexane extract (0.668±0.004 and 46.30%) > ethanol extract (0.301±0.003 and 20.80%). However standard drug had strong reducing power in comparison with all test samples. The reducing power showed good linear relation of pure compound piperine ($r^2 = 0.867$), hexane extract ($r^2 = 0.968$) and ethanol extract ($r^2 = 0.953$).

Table 3. Reducing power capacity of piperine, hexane and ethanol extracts of *Piper nigrum*

Concentration µg/ml	**Absorbance at 700 nm**			
	Standard Drug (Ascorbic acid)	**Piperine**	**Hexane Extract**	**Ethanol Extract**
100	0.106 ±0.005	0.045±0.0007	0.078±0.004	0.050±0.0007
250	0.514±0.011	0.066±0.001	0.245±0.009	0.135±0.002
500	1.026±0.006	0.113±0.002	0.430±0.0007	0.150±0.004
1000	1.441±0.094	1.039±0.015	0.668±0.004	0.301±0.003

The results are means of triplicate estimations, ± SEM

Table 4. Percentage of reducing power capacity of piperine, hexane and ethanol extracts of *Piper nigrum*

Concentration µg/ml	**Percentage of reducing power capacity**			
	Standard Drug (Ascorbic acid)	**Piperine**	**Hexane Extract**	**Ethanol Extract**
100	7.30	3.0	5.4	3.4
250	35.60	4.5	17.0	9.3
500	71.30	7.8	30.0	10.4
1000	-	72.1	46.3	20.8

Percentage of reduction power calculated by absorbance of standard (ascorbic acid) at maximum concentration tested

Hepatocytes integrity markers

Control group

Blood serum of control group, subjected to biochemical analysis. Liver biochemistry processed by analysis of serum levels of enzymes (ALP, ALT, γ-GT, DB and TB) these enzymes were found not elevated when compared to toxicant group (Table 5).

Toxicant group

Significant reduction in animal's body weight and excised liver weight were observed in CCl_4 administered group. Enzymatic activity of major intracellular enzyme ALP and ALT, γ-GT, DB and TB (Table 5) were substantially increased to 2.4, 3.3, 4.6, 21.3 and 9.69 folds as compared to normal control group.

Standard drug group (Prophylactic model)

Pretreatment with standard drug in high concentration (16.13 mg/kg body weight) significantly prevented the CCl_4 induced hepatic damage level of ALP, ALT, γ-GT, DB and TB up to about 75.24%, 78.05%, 63.91%, and 93.24% when compared to CCl_4 and control normal group. Although at low concentration 5.37 mg/kg body weight the release of liver enzyme in blood serum were noted more than control but less than toxicant group. Moreover pretreatment of standard drug at a dose of 10.75 mg/kg body weight showed significant reduction ($P<0.01$) in ALT and TB, while release of ALP, γ-GT and DB observed less than normal as well as toxicant group (Table 5).

Tested samples group (Prophylactic model)

Hepatoprotective effect of pure compound piperine at high dose of 100 mg/kg body weight when compared to toxicant and normal control group it was significantly inhibited the serum level of ALP to about 77.64% however serum levels of ALT, γ-GT, TB and DB were restored back to normal level. Furthermore hexane extract at dose of 150 mg/kg body weight significantly affect the serum ALP, ALT, γ-GT and DB levels to about 62.45%, 67.43%, 78.26% and 93.87% respectively. Whereas administration of ethanol extract 150 mg/kg body weight showed significant alteration in ALP, ALT and DB levels to 52.82%, 64.60% and 91.83% respectively. In addition treatment with pure compound piperine at 25 and 75 mg/kg body weight while hexane and ethanol extracts treatment at 50 and 100 mg/kg body weight were reduced the increased hepatic serum enzymes and effect were noted protective when compared to toxicant group (Table 5) but no significant changes in serum enzymes levels were observed as compared to control group.

Standard drug group (Therapeutic model)

Treatment with standard drug after liver toxicity induced by CCl_4 revealed significant reduction in the levels of hepatic enzymes in dose dependent manner when compared to toxicant group. Further comparison to control group, standard drug was not presented the potential in reducing of serum enzymes levels (Table 6).

Tested samples group (Therapeutic model)

Therapeutic hepatoprotective potential of piperine, hexane and ethanol extracts against CCl_4 administration were demonstrated in (Table 6). High dose treatment 100 mg/kg body weight of piperine led to significant reduction in levels of ALT, ALP and TB up to 72.33%, 82.60% and 85.71% respectively. Low tested dose of piperine 25 mg/kg body weight exhibited minimal level of serum enzymes suppression in comparison with toxicant group although found effective in comparison with control group. Hexane and ethanol extracts at doses of 50 and 100 mg/kg of body weight caused reduction in ALP, ALT and TB serum levels in comparison with toxicant group while moderate reduction of serum enzymes were observed in comparison to control group. The toxic effect of CCl_4 was controlled in animals treated with 150 mg/kg body weight of hexane and ethanol extracts led to lowering DB and TB up to 93.87% and 80.95% respectively as compared to toxicant group.

Histopathological study

Control group

Histopathological results of healthy control liver tissues (Table 7 and Fig.1) indicated normal liver lobular architecture and cell structure; each hepatocyte had distinctly rounded nuclei. Connective tissues around the central vein are clearly seems. In addition portal vein, bile duct and hepatic arteries showed typical cell structure.

Toxicant group

This examination of liver section obtained from CCl_4 hepatoxicity showed marked congestion of portal tract (severally diffused), hepatocellular damage with prominent bridging fibrosis and architectural distortion; interlobular damaged scattered fatty degeneration and necrosis were presented in (Table 7 and Fig. 2).

Standard drug group (Prophylactic model)

Pretreatment of standard drug at high dose 16.13 mg/kg body weight prevented the CCl_4 induced hepatic damage when compared to toxicant and control groups. Normal hepatic cells architecture, central and portal vein areas confirming the prevented strength of the standard drug at selected dose (Fig. 3). Although treatment with less concentrations 5.37 and 10.75 mg/kg body weight presented minimal inflammation that introduced occasional spotty necrosis (Grade 1) and stage 1 fibrosis of portal tract (Table 7).

Tested samples group (Prophylactic model)

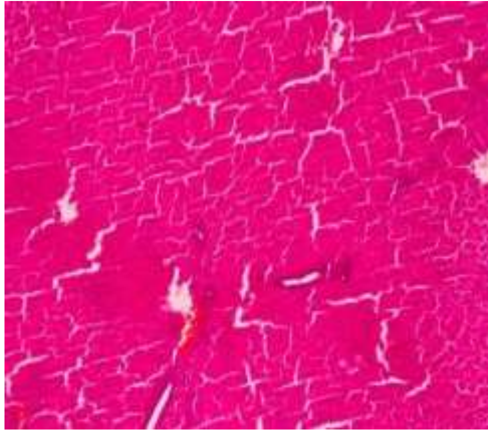
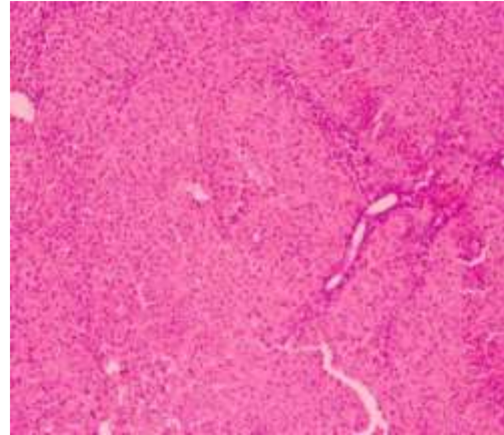
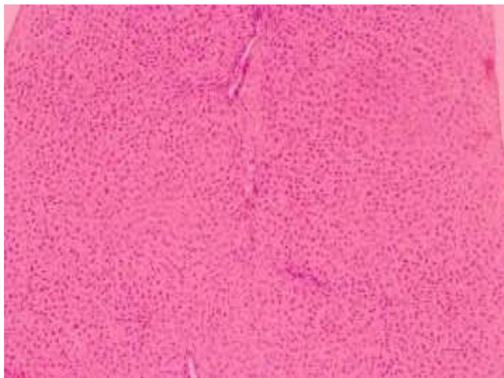
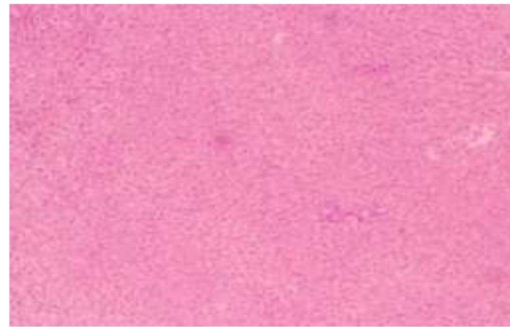
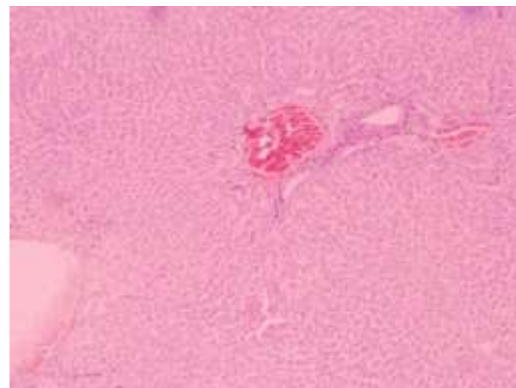
Animals pretreatment with pure compound piperine at 100 mg/kg body weight showed in Table 7 and Fig. 4. Furthermore, hexane extract at a dose of 150 mg/kg body weight (Table 7) and (Fig. 5) also confirmed the normal hepatocellular morphology from damaging effect of CCl_4, hepatocytes and have round nucleus, flat endothelial cells around the central portal vein. On other hand ethanol extract at a dose of 150 mg/kg body weight showed presences of fibrosis, moderately inflamed micro and macro fatty vascular hepatocytes and focal necrosis (Table 7 and Fig. 6).

Standard drug group (Therapeutic model)

The liver section of rats treated with high and moderate doses such as 16.13 and 10.75 mg/kg body weight presented visible bile duct, portal vein, lobular central vein and distinct hepatic cells structure (Fig. 7). However less dose treatment 5.37 mg/kg body weight degeneration and disarrangement of hepatocytes and cirrhosis of portal vein with mild fatty changes in liver were observed (Table 8).

Tested samples group (Therapeutic model)

The section of the liver taken from the animals treated with low doses of piperine, hexane and ethanol extracts exhibited the appearance of necrosis and ballooning generation, different stages and grades of fibrosis and inflammation observed in hepatocytes. Although, occasional degeneration of hepatocytes along with intact bile duct, portal vein and lobular central vein was observed in a liver section treated with high doses of piperine, hexane and ethanol extracts (Fig. 8, 9, 10 and Table).

 Figure 1	 Figure 2
 Figure 3	 Figure 4
Figure 5	 Figure 6

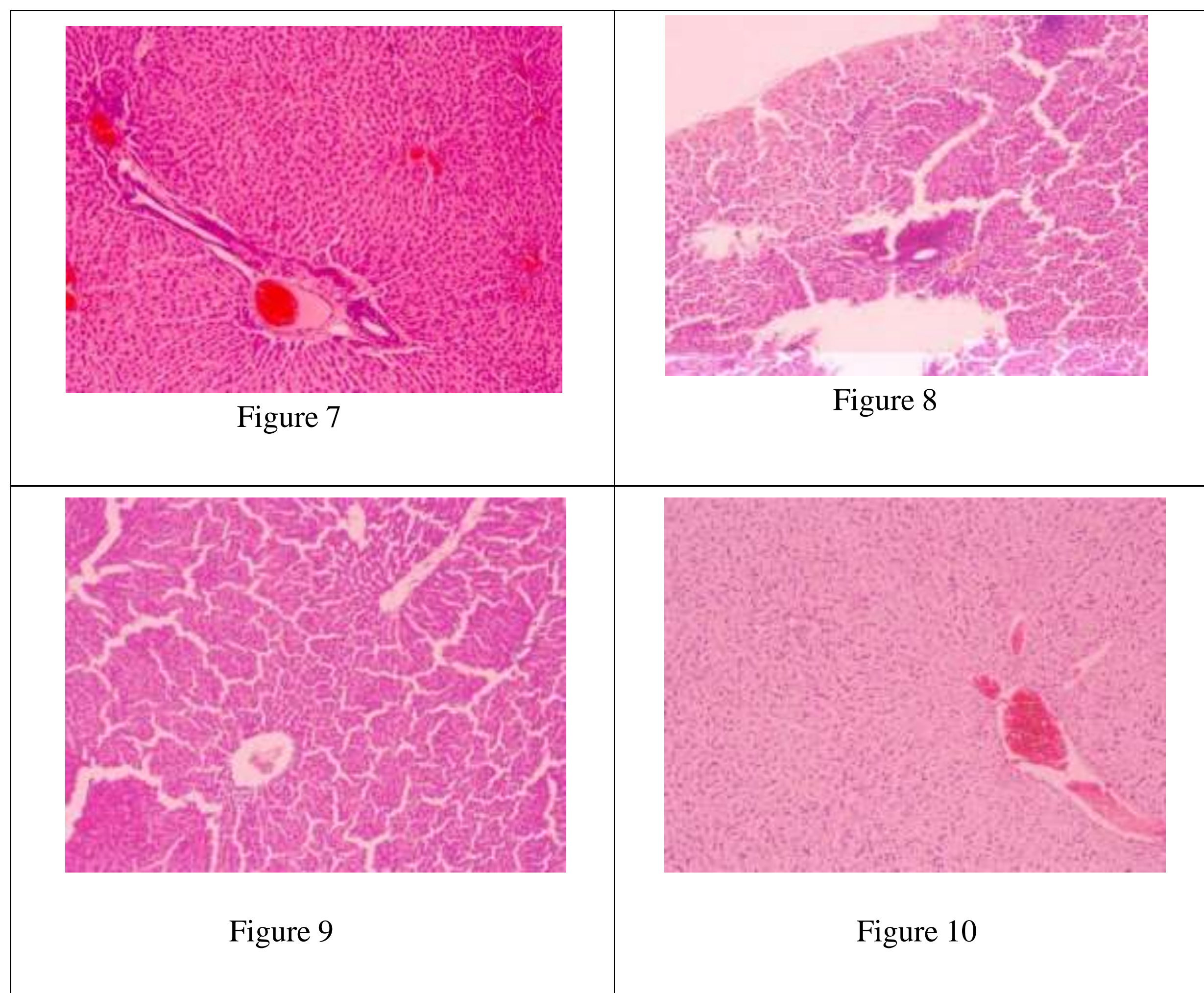


Figure 1 Histopathological micrograph of a normal rat (Control Group)

Figure 2 Histopathological micrograph of a rat treated with CCl_4

Figure 3 (Prophylactic model) Histopathological micrograph of rat liver section treated with 16.13 mg/kg standard drug and CCl_4

Figure 4 (Prophylactic model) Histopathological micrograph of rat liver section treated with 100 mg/kg pure compound piperine and CCl_4

Figure 5 (Prophylactic model) Histopathological micrograph of rat liver section treated with 150 mg/kg hexane extract of *Piper nigrum* fruit and CCl_4

Figure 6 (Prophylactic model) Histopathological micrograph of rat liver section treated with 150 mg/kg ethanol extract of *Piper nigrum* fruits and CCl_4

Figure 7 (Therapeutic model) Histopathological micrograph of rat liver section treated with 16.13 mg /kg standard drug and CCl_4

Figure 8 (Therapeutic model) Histopathological micrograph of rat liver section treated with 100 mg/kg pure compound piperine and CCl_4

Figure 9 (Therapeutic model) Histopathological micrograph of ratliver section treated with 150 mg/kg hexane extract of *Piper nigrum* fruits and CCl_4

Figure 10 (Therapeutic model) Histopathological micrograph ofrat liver section treated with 150 mg/kg ethanol extract of *Piper nigrum* fruits and CCl_4

Table 5. Effect of *Piper nigrum* on biochemical parameters relating to hepatoprotective properties (prophylactic model)

Compound and Extracts	Dose mg/kg	ALP (IU/ L)	ALT (IU/ L)	γ-GT (IU/ L)	DB (mg/ dl)	TB (mg/ dl)
Piperine	25	400.80±0.001**	81.80±0.008**	2.00±0.061	0.12±0.004**	0.24±0.001**
	75	287.00±0.001**	49.60±0.001**	0.400+0.178	0.08±0.016	0.34±0.003**
	100	143.60±0.006**	40.66±0.013	0.80±0.099	0.04±0.178	0.260±0.003**
Piper nigrum (Hexane Extract)	50	373.0±0.011	61.20±0.004**	3.60±0.374	0.066±0.184	0.90±0.016
	100	246.60±0.007**	50.33±0.067	3.200±0.300	0.06±0.070	0.500±0.020
	150	252.40±0.001**	46.00±0.001**	1.00±0.142,	0.06±0.070	0.38±0.001**
Piper nigrum (Ethanol Extract)	50	439.00±0.003**	100.00±0.218	2.20±0.040	0.72±0.109	0.40±0.019
	100	389.20±0.006**	67.00±0.004**	1.60±0.003**	0.14±0.025	0.36±0.001**
	150	303.00±0.001**	50.00±0.001**	1.40±0.135	0.08±0.016	0.36±0.011
Standard drug (Jetepar)	5.37	279.80±0.001*	59.33±0.002**	4.00±0.099*	0.08±0.016	0.42±0.001*
	10.75	199.33±0.025	35.66±0.004**	4.00±0.208	0.08±0.016	0.40±0.002**
	16.13	159.00±0.011**	31.00±0.009**	1.66±0.199	0.066±0.03	0.38±0.024
Control	-	258..25±0.006	42.00±0.007	1.00±0.034	0.046±0.033	0.13±0.002
Toxicant (CCl_4)	1.5 ♦	642.30±0.002	141.25±0.017	4.60±0.006	0.98±0.001	1.26±0.043

ALP= Alkaline phosphatase, ALT= Alanine aminotransferase, γ--GT= Gamma-glutamyl transpeptidase, DB= Direct bilirubin, TB= Total bilirubin
IU/ l= International units per liter, mg/dl= Milligram per deciliter
All values are given in Mean ± SEM, n=5, *P<0.001 and **P<0.01 significant to toxicants
♦Dose of toxicant in ml/kg body weight

Compound and Extracts	Dose mg/kg	ALP (IU/ L)	ALT (IU/ L)	γ-GT (IU/ L)	DB (mg/ dl)	TB (mg/ dl)
Piperine	25	541.80±0.004**	54.60±0.001**	1.60±0.003**	0.08±0.016	0.50±0.001**
	75	253.80±0.003**	50.40±0.001**	1.40±0.005**	0.06±0.070	0.38±0.001**
	100	224.80±0.001**	39.08±0.018	0.80±0.099	0.06±0.070	0.18±0.021
Piper nigrum (Hexane Extract)	50	403.00±0.010	91.40±0.006**	2.00±0.022	0.08±0.016	0.70±0.001**
	100	336.60±0.001**	52.00±0.001**	1.60±0.306	0.08±0.020	0.30±0.001**
	150	265.60±0.001**	47.20±0.001**	1.00±0.034	0.08±0.016	0.24±0.001**
Piper nigrum (Ethanol Extract)	50	373.20±0.001**	63.80±0.002**	2.40±0.109	0.12±0.004**	0.46±0.001**
	100	345.80±0.002**	70.80±0.003**	2.20±0.004**	0.08±0.016	0.36±0.024
	150	261.80±0.002**	59.20±0.021	1.20±0.109	0.08±0.016	0.32±0.020
Standard drug (Jetepar)	5.37	275.200±0.003**	77.000±0.007**	4.000±0.041	0.400±0.041	0.520±0.012
	10.75	263.000±0.008**	63.600±0.001**	3.600±0.029	0.200±0.116	0.360±0.001
	16.13	228.000±0.012	59.400±0.002**	0.600±0.070	0.120±0.004**	0.160±0.056
Control	-	258..25±0.006	42.00±0.007	1.00±0.034	0.046±0.033	0.13±0.002**
Toxicant (CCl_4)	1.5♦	642.30±0.002	141.25±0.017	4.60±0.006	0.98±0.001	1.26±0.043

Table 6. Effect of *Piper nigrum* on biochemical parameters relating to hepatoprotective properties (therapeutic model)

ALP= Alkaline phosphatase, ALT= Alanine aminotransferase, γ--GT= Gamma-glutamyl transpeptidase, DB= Direct bilirubin, TB= Total bilirubin
IU/ l= International units per liter, mg/dl= Milligram per deciliter
All values are given in Mean ± SEM, n=5, *P<0.001 and **P<0.01 significant to toxicants
♦Dose of toxicant in ml/kg body weight

Table 7. Hepatic histopathological findings of *Piper nigrum* (prophylactic model)

Compound and Extracts	Dose mg/kg	Portal Tract				Lobules					Loss of central vein
		Fibrosis (Stage)	Inflammation (Grade)	Bile duct	Portal vein	Lobular damage	Hepatocytes		Fatty liver		Finding
							Degeneration	Necrosis	Micro	Macro	
Piperine	25	3	3	Intact	Intact	-ve	-ve	-ve	+ve	+ve	Intact
	75	2	2	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	100	0	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Piper nigrum (Hexane Extract)	50	2	2	Intact	Intact	-ve	-ve	Focal	-ve	-ve	Intact
	100	1	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	150	0	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Piper nigrum (Ethanol Extract)	50	2	3	Intact	Intact	-ve	+ve	Focal	Moderate +ve	Moderate +ve	Intact
	100	1	3	Intact	Intact	+ve	Focal	Focal	+ve	-ve	Intact
	150	1	3	Intact	Intact	+ve	Focal	Focal	-ve	-ve	Intact
Standard Drug (Jetepar)	5.37	1	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	10.75	1	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	16.13	0	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Control	-	0	0	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Toxicant (CCl_4)	1.5♦	3	4	Intact	Intact	+ve	+ve	+ve	+ve	Mild +ve	Intact

Table 8. Hepatic histopathological finding of *Piper nigrum* (therapeutic model)

Compound and Extracts	Dose mg/kg	Portal Tract					Lobules				Loss of Central vein
		Fibrosis (Stage)	Inflammation (Grade)	Bile duct	Portal vein	Lobular damage	Hepatocytes		Fatty liver		Finding
							Degeneration	Necrosis	Micro	Macro	
Piperine	5	4	3	Intact	Intact	+ve	+ve	Focal	+ve	+ve	Intact
	75	2	2	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	100	1	2	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Piper nigrum (Hexane Extract)	50	1	1	Intact	Intact	-ve	-ve	-ve	+ve	+ve	Intact
	100	1	2	Intact	Intact	-ve	+ve	-ve	-ve	-ve	Intact
	150	0	1	Intact	Intact	Minor+ve	-ve	-ve	-ve	-ve	Intact
Piper nigrum (Ethanol Extract)	50	2	3	Intact	Intact	-ve	-ve	-ve	+ve	+ve	Intact
	100	0	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	150	0	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Standard Drug	5.37	4	3	Focal	Intact	-ve	+ve	-ve	Mild +ve	Mild +ve	+ve
	10.75	0	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
	16.13	1	1	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Control	-	0	0	Intact	Intact	-ve	-ve	-ve	-ve	-ve	Intact
Toxicant (CCl_4)	1.5♦	3	4	Intact	Intact	+ve	+ve	+ve	+ve	Mild +ve	Intact

♦Dose of toxicant in ml/kg body weight

DISCUSSION

Free radicals are extremely reactive and unstable molecules that are generated during normal cellular function within the body naturally as a byproduct of metabolism (oxidation), or by exposure to toxins. The life expectancy of free radicals is just for a second, but during that time it involved in natural cellular activities in a controlled manner, while unbounded could damage DNA, lipids, proteins, and other important biological molecules. They are the main contributors of oxidative stress; once free radicals have been produced they are allowed to cause damage and development of multiple disorders [21]. Various research studies consequences demonstrated that there is a substantial concern in searching of the naturally occurring anti-oxidants specially extracted from plants sources, with the strength to save the human organs damages against free radical. Literature data indicate that anti-oxidant compounds inhibit the activity of free radicals which have been associated in the pathogenesis of different diseases including dementia leads to Alzheimer, cardiovascular, metastasis and liver disorders. Subsequently, the exploration of novel anti-oxidant agents has caught more scientific interest. It is previously reported that the plants rich in secondary metabolites, including alkaloids, flavonoids, tannins and phenol. Which are strong anti-oxidants due to their redox action and chemical structures and it is mainly depends onthe presence of free OH groups, specifically 3-OH [22]. In this study phytochemical screening demonstrated the presences of different types of alkaloids, flavonoids; phenols; tannins and steroidal contents, and the analysis suggest that ethanol extract of *Piper nigrum* fruits contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavengethe potential damage.

In anti-oxidant assay, the reducing power ability of ethanol and hexane extracts along with pure compound piperine was analyzed, in comparison with ascorbic acid as a standard. This assay is frequently used to detect the ability of an anti-oxidant compound to donate an electron. The

presence of anti-oxidants in extracts and pure compound followed the reduction of the ferric cyanide complex $Fe^{3}+$ to the ferrous cyanide form $Fe2^{+}$, thereby transition of solution into various shades from green to blue, depending on the reducing power capacity of the extracts and compound [23]. Strong reducing agents formed Perl's Prussian blue color and absorbed at 700 nm. (Table 2 and 3) showed the reducing activities of ethanol, hexane extracts and pure compound piperine. The higher the absorbance of the reaction mixture, higher would be the reducing power. Ethanol and hexane extracts showed some degree of electron donation. Moreover the anti-oxidant results depicted that reducing power of test samples increased with an increased in the concentrations. While piperine possessed highest degree of Fe3+ reduction at highest concentration among all test samples but less than standard drug. The reducing power of standard ascorbic acid was found higher than all the tested samples. It has been reported that the reducing power of substances is probably because of their hydrogen donating ability that can damage hundreds of molecules [24]. Pure compound piperine therefore, have high amount of reductions than ethanol and hexane extracts. Hence, pure compound piperine act as electron donors and could react with free radicals to convert them into more stable products and then terminate the free radical chain reactions. Apart from this study, piperine inhibited oxidative damage levels that could potentially be related with its therapeutic effect on free radicals and reactive oxygen species by inhibition of lipid per oxidation and enhancement of glutathione [25, 26] and this research proved strong anti-oxidant potential of *Piper nigrum.*

Carbon tetrachloride (CCl_4) is one most common hepatotoxin used in experimental study of liver diseases. Series of mechanisms involved in the hepatotoxic effect of CCl_4, first is oxidative damage through free radical generation. Ingestion of CCl_4 caused bioconversion of cytochrome P450 to highly reactive trichloromethyl (CCl_3) as a free radical, which binds with the lipoprotein of endoplasmic reticulum and persuade membrane lipid per oxidation that may interrupt $Ca^{2}+$ homeostasis that would incorporate with the production of toxins and may leads to many

morphological and functional hepatocellular injury [22].Current *in-vivo* study indicated that a single oral administration of CCl_4 caused per oxidation in adipose tissues resulting in fatty infiltration of hepatocytes. The increased levels of serum bilirubin reflected the depth of jaundice and the increased in ALP and ALT levels were clear indication of cellular leakage and loss of functional integrity of cell membrane [2, 3]. Marked elevation in the serum enzymes might be due to release of these enzymes from cytoplasm into the blood stream rapidly after rupture of the plasma membrane and cellular damage. Significant hepatoprotection were exhibited by piperine, hexane and ethanol extracts in prophylactic model. However treatment with pure compound piperine provides hepatoprotective action both in prophylactic and therapeutic models in a dose dependent manner towards normal. This reduction in levels of ALT is an indication of hepatic cell regeneration. While decrease level of ALP and bilirubin showed that tested samples stabilized the biliary function. In addition tested samples protected both plasma membrane and liver cells against damage thereby decreasing the leakage of serum enzymes into blood circulation and modulate ALP, ALT and bilirubin hence it can be proposed to beneficial in hepatocellular damage, obstructive jaundice and hepatitis conditions. This result also supported by histopathological studies, negligible damages of portal tract, lobules and central vein were seen in animals. Hence it may be possible that the mechanism of hepatoprotection of extract is due to its anti-oxidant effect.

Piperine is one of the plant derived alkaloid isolated from *Piper nigrum* fruit containing a phenolic hydroxyl group. Piperine obstructs the oxidative damage levels that could potentially be referred with its reducing effect of free radicals and reactive oxygen species [26]. The anti- oxidant potential of piperine evolved by the reduction of lipid per oxidation and increases the glutathione synthesis and transport [25]. Previous studies have indicated that piperine anti-oxidant property is linked with the molecule's carbon-oxygen five-membered rings and amide structure. One of the study reported that piperine endure demethylation of its carbon-oxygen five-membered rings during metabolism in the human body, primarily generating metabolites with a bisphenol hydroxyl

structure [27]. The resultant synthesis of phenolic hydroxyl groups strengthen the anti-oxidant activity of piperine [28] and [21] This study synthesized novel piperine derivatives for use as potential anti-oxidant agent candidates and also opens newer views on the potential efficacy of piperine in protecting tissues from per oxidative damage.

The efficacy of the hepatoprotective drug is depending on its capacity of either reducing the harmful effect or maintaining the normal hepatic physiology which have been disturbed by hepatotoxin. Serum level of transaminase returns to normal with the healing of parenchyma and regeneration of hepatocytes while effective control of bilirubin level point towards an early improvement in the secretary mechanism of hepatic cells.

CONCLUSION

Results of present study indicated that *Piper nigrum* fruit possessed good hepatoprotection evidence by decreasing serum ALT and ALP levels significantly coupled with anti-oxidant and anti-inflammatory potential [29]. Anti-oxidant activity recognized due to main alkaloid (piperine) that have radical scavenging and anti-lipo per oxidation effect. The membrane stabilizing property is mainly as responsible for its hepatoprotection. Therefore results of this study may be applied a guideline to CCl_4 hepatotoxic mechanism with valuable data for formulating anti-hepatotoxic drugs.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Declaration of Competing Interest

All the authors declared that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement:

The animal study reviewed and approved by Board of Advanced Studies and Research, University of Karachi BASR/No./0265/ Pharm.

References

1. Kamble MB, Dumbre RK, Rangari VD. *Hepatoprotective activity studies of herbal formulations.* International Journal of Green Pharmacy.2008; 2 (3): 147-151; doi:10.4103/0973-8258.42731.

2. Argal A, Dwivedi A. *Evaluation of hepatoprotective activity of Calotropisgigantea R.Br. flowers.* Ethno botanical Leaf. 2010; 4 (14): 427-434.

3. Nirwane AM, Bapat AR. *Effect of methanolic extract of Piper nigrum fruits in ethanol CCl_4 induced hepatotoxicity in Wistar rats*. Der Pharmacia. Lettre. 2012; 4 (3): 795-802.

4. Khan S, Mirza KJ, Anwar F, Abdin MZ. *Development of RAPD markers for authentication of Piper nigrum* L. Environment and we An International Journal of Science and Technology. 2010; 5: 47-56.

5. Tasleem F, Azhar I, Ali SN, Perveen S, Mahmood ZA. *Analgesic and anti-inflammatory activities of Piper nigrum* L. Asia Pacific Journal of Tropical Medicine. 2014; *7*:S461-S468.

6. Al-Shahwany AW. *Alkaloids and phenolic compound activity of Piper nigrum against some human pathogenic bacteria.* Journal of Biomedicine and Biotechnology. 2014; 2(1):20-28.

7. Tiwari A, Mahadik KR, Gabhe SY. *Effect of piperine and its analogs on pharmacokinetic properties of sorafenib tosylate: Bioanalytical method development and validation.* Journal of Applied PharmaceuticalSciences.2020;10(12):001-012.http://doi:10.7324/JAPS.2020.101201.

8. Harborne, JB. *Phytochemical methods*, London. Chapman and Hall, Ltd. 1973: p. 49-188.

9. Sofowara A. *Medicinal plants and Traditional medicine in Africa*. Spectrum Books Ltd,

Ibadan, Nigeria, 1993: p. 289.

10. Trease, G.E. and Evans,W.C.(1989). *Pharmacognsy*(11th ed.). Brailliar Tiridel Can. Macmillian.

11. Anu S, Deepashree GHM, Shruthi S, Singh V, Bole S, Balu S, et al. *Phytochemical screening, antioxidant and antibacterial activity of Piper longum*. International Journal of Current Research. 2013; 5(2):148-152.

12. Bukhari SB, Bhanger MI, Memon S. *Antioxidative activity of extracts from Fenugreek* (*Trigonella foenum-graecum*). Pakistan journal of Analytical and Environmental Chemistry. 2008; 9 (2): 78-83.

13. Doss A, Pugalenthi M. *Evaluation of antioxidant activity and phytochemical screening of Malus domestica borkh (apple) and Phaseolus vulgaris L.(green beans).* Journal of Pharmaceutical Science Innovation. 2012; 1 (3): 1-4.

14. Rohman A, Riyanto S, Yuniarti N, Saputra W R, Utami R, Mulatsih W. *Antioxidant activity, total phenolic, and total flavaonoid of extracts and fractions of red fruit* (*Pandanus conoideus* Lam.). International Food Research Journal. 2010; 17: 97-106.

15. Lodhi Singh H, Pant K, Hussain Z. *Hepatoprotective effects of Calotropisgigantea extract against carbon tetrachloride induced liver injury in rats*. Acta pharmaceutica. 2009; 59 (1): 89-96; doi: 10.2478/v10007-009-0002-2.

16. Edwards CRW, Bouchier IAD. *Davidson's Principles and Practice of Medicine*. Publisher: *ELBS* with Churchill Livingstone man group Ltd. Hong Kong, 1991:p. 600-745.

17. Alqasoumi SI, Farraj AI, Abdel-Kader MS. *Study of the hepatoprotective effect of Juniperusphoenicea constituents.* Pakistan Journal of pharmaceutical Sciences. 2013; 26(5):999-1008.

18. Batts KP, Ludwig J. *An Update on Terminology and Reporting*. The American Journal of surgical Pathology. 1995; 19 (12): 1409-1417.doi: 10.1097/00000478-199512000-00007.

19. Gelow JM, Desai AS, Hochberg CP, Glickman JN, Givertz MM, Fang JC. *Clinical predictors of hepatic fibrosis in chronic advanced heart failure.* Circulation Heart Failure. 2010; 3 (1): 59-64; doi: 10.1161/CIRCHEARTFAILURE.109.872556.

20. Ganesh P, Suresh KR, Saranraj P. *Phytochemical analysis and antibacterial activity of pepper (Piper nigrum L.) against some human pathogens.* Central European Journal of Experimental Biology. 2014; 3 (2): 36-41.

21. Qin B, Yang K, Cao R. Synthesis and Antioxidative Activity of Piperine derivatives Containing Phenolic Hydroxyl. Journal of Chemistry. 2020; 2020, ID 2786359, 9.

22. Laouar A, Klibet F, Bourogaa E, Benamara A, Boumendjel A, Chefrour A, et al.

Potential antioxidant properties and hepatoprotective effects of Juniperus phoenicea berries against CCl4 induced hepatic damage in rats. Asian Pacific Journal of Tropical Medicine. 2017; 10 (3): 263-269. http://dx.doi.org/10.1016/j.apjtm.2017.03.005.

23. Ferreira IC, Baptista P, Vilas-Boas M, Barros L. *Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal individual cap and stipe activity*. Journal of Food Chemistry. 2007; 100 (4):1511–1516.

24. Shimada K, Fujikawa K, Yahara K, Nakamura T. *Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion.* Journal of Agricultural and Food. 1992; 40(6): 945–948.

25. Khajuria A, Thusu N, Zutshi U, Bedi KL. *Piperine modulation of carcinogen induced oxidative stress in intestinal mucosa.* Molecular and Cell Biochemistry. 1998; 189 (1-2):113–118; doi: 10.1023/a: 1006877614411.

26. Verma N, Bal S, Gupta R, Yadav A. *Antioxidative effects of piperine against Cadmium-induced oxidative stress in cultured human peripheral blood lymphocytes*, Journal of Dietary Supplements. 2018; 17 (1): 41–52. doi:10.1080/19390211.2018.1481485.

27. Velpandian T, Jasuja R, Bhardwaj RK, Jaiswal J, Gupta SK. *Piperine in food interference in the pharmacokinetics of phenytoin.* European Journal of Drug Metabolism and Pharmacokinetic. 2001; 26 (4): 241–247. doi: 10.1007/BF03226378.

28. Adjimani JP, Asare P. *Antioxidant and free radical scavenging activity of iron chelators.* Toxicol0gy Report. 2015; 11(2):721–728. doi: 10.1016/j.toxrep.2015.04.005.

29. Tasleem F. Biomedical analysis on phytopharmaceuticals. 2015. Unpublished dissertation for the fulfillment of the degree of Doctor of Philosophy, Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi, Pakistan.